ACTIVE COMPOUNDS FROM TULSI INHIBITS BENZO [A]PYRENE MEDIATED CYTOTOXICITY:*in vitro&in silico* Analysis

A THESIS SUBMITTED IN PARTIAL FULFILLMMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

LIFE SCIENCE

SUBMITTED TO

NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA

BY

SUBHALAKSHMI SAMAL

ROLL NO 412LS2058

UNDER THE SUPERVISION OF

DR.SUJIT KUMAR BHUTIA

DEPARTMENT OF LIFE SCIENCE

NATIONAL INSTITUTE OF TECNOLOY , ROURKELA

PIN-769008

Dr. Sujit Kumar Bhutia
Assistant Professor.
Department of life sciences
National institute of Technology Rourkela
Rourkela-769008, Odisha, India
Ph-91-661-2462686
Email:sujitb@nitrkl.ac.in, bhutiaask@gmail.com

Ref. No.
Date:

CERTIFICATE

This is to certify that the thesis entitled **"Active compounds from tulsi inhibits benz[a]pyrene mediated cytotoxicity: *in vitro* & *in silico* Analysis"** which is being submitted by Ms. Subhalakshmi Samal, Roll No.- 412LS2058, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

Sujit K Bhutia

DECLARATION

I do hereby declare that the Project Work entitled **"Active compounds from tulsi inhibits benz[a]pyrene mediated cytotoxicity: *in vitro* & *in silico* Analysis"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Sujit Kumar Bhutia, Asst. Professor, Department of Life Science, National Institute of Technology, Rourkela, Odisha.

Date:

Place: Subhalakshmi Samal

ACKNOWLEDGEMENTS

I have a feeling of a dream come true. Inventing and learning new things is the things which I got to know through this research. Failures are the pillars to success is well proved in the research work .I would express my gratitude to almighty invisible power to give me mental support to make this work a grand success.

I would like to thank my Parents and family members who got pains as well as happiness to get me in this prestigious institute for studying this course with a great variety of curriculum which helps us to be confident of ourselves. I am very thankful to my supervisor, guide and philosopher, Dr. SujitKumarBhutia Assistant Professor of NIT Rourkela who helped in very prospect of the work . His constant motivation inspired me lot to adventure in the field of research and preparation of the thesis.

I am grateful to Dr.S.K.Patra HOD Dept. of Life sci, my Faculty Advisor Dr. BibekanandaMallick, Dr.RasuJayabalan, Dr.Surajit Das and Dr. SumanJha for their valuable support throughout the period of my stay at this institute.

I am highly obliged to my mentor DurgeshNandini Das for her faith, affection and encouragement to me throughout this project to make it a success along with Prashanta Kumar Panda, SubhadipMukhopadhay, Niharika Sinha and Prajna P. Naik. Last but not the least my best lab pals who supported me through team work and their affection cherished my heart.

“United we stand Divided we fall.”

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ABSTRACT

Environmental Pollution is the one of the major problem of the hour. In this fast moving scenario, pollution both industrial and air plays a major role in affecting living beings. These pollutants mainly consist of mainly organic pollutants and particulate matter. Polycyclic Aromatic Hydrocarbons (PAH) are the groups of organic pollutants that are ubiquitously present, resistant to degradation and can stay for a longer time in the atmosphere.They are known as mutagen, carcinogen and tetratogen. One such PAH is Benzo[a]pyrene, which is first pro-carcinogen to be discovered which causes skin cancer and respiratory problems etc. Benzo[a]pyrene is a five membered ring which is basically produced from incomplete combustion of fossil fuels, exhaust from automobiles and industries. The toxicity of Benzo[a]pyrene is the result of bioactivation of B[a]P to diolepoxides by monooxygenase enzymes i.e. CYP450 enzyme systems in human body. Diol epoxides are DNA adducts which mutate the p53 tumor suppressor gene leads to tumor. Hence these enzymes are inhibited by active compounds extracted from Black tulsi. They are eugenol, carvacrol, elemene, caryophyllene and linalool. In this study we have reported that active compounds of tulsi bind to cytochrome P450 enzyme active site and inhibit those from binding benzo[a]pyrene. By docking studies done in Hex software has proved that active compounds of tulsi bind to CYP450. Also not only *in-silico* studies but also *in-vitro* analysis like MTT assay, DAPI staining and EROD assay was done to confirm our topic.

Keywords: particulate matter, benzo[a]pyrene, CYP450, Tulsi

INTRODUCTION

Pollution is the major problem of the today‘s first moving industrialization and globalization .Air Pollution plays a major role in affecting human health as well as environment. The hazardous chemicals producing from the industries, transportation and burning of fossil fuels are the major air pollutant causing various diseases to human being and other organisms. Air pollutants are mainly gases.The pollutants include gaseous substances such as sulphur oxide, nitrogen dioxide, and carbon monoxide, particulate matter including organic, inorganic matter, nitrogen compounds, sulphur compounds, polycyclic aromatic hydrocarbons (PAHs), several heavy metals, and radio nuclides. Air polluting industries include thermal power plants, iron and steel plants, smelters, foundries, stone crushers, cement plants, refineries, shoe industry, plastic industry, chemicals and petrochemical plants. The main source of gaseous pollutants are from incomplete burning of fossil fuels, (Kampa et al, 2007) next is automobiles and air from chimneys of factories. The second pollutants are the Organic pollutants. They form a toxic group of chemicals which persist for a long interval of time in the environment.Their effects are amplified as they move up through the food chain which include pesticides, dioxin and furans. (Schecter et al, 2006). Dioxins are those substances which are formed during incomplete combustion and materials containing chlorine (e.g. plastics). Most dioxins are present in plants are from air or pesticides and these enter the food chain where they accumulate due to their ability of binding to lipids. The third groups of pollutants are Particulate Matter which consists of complex and varying mixturesof particles suspended in the air, which vary in size and composition. They are produced by a variety ofnatural and anthropogenic activities (Poschl et al, 2005). Major sources are from factories, atomic power plants, refuse incinerators, motor vehicles, construction sites, firesand natural windblown dust. The size of the particles varies from 2.5-10 microns for

aerodynamic diameter smaller than2.5mm and 10mm respectively (Kampa et al, 2007). Some other pollutants are heavy metals like arsenic, cadmium, lead, chromium, copper; nickel, selenium and zinc are emitted from combustion and industrial activities like metals works and smelters. The composition of PM varies, as they can absorb and transfer a multitude of pollutants. In addition, the metal content, the presence of PAHs and other organic components such as endotoxins, mainly contribute to PM toxicity (Knaapen, et al., 2002). Benzo [a]pyrenewhich is one of the priority pollutant, heavy metals can reside in or be attached to the particulate matter. The last pollutants are known to be Volatile Organic Compounds. It consists of polycyclic aromatic hydrocarbon. These compounds are as follows: Naphthalene, acenaphthylene, fluorene, anthracene, fluoranthene, chrysene, benzo[a]pyreneand dibenzo (a, h) anthracene. The high mortality indicates by skin and lung cancers are the most notable examples of the carcinogenic potential of PAH compounds. (Mastral et al, 2000)Polycyclic Aromatic Hydrocarbonsare fused aromatic rings and do not contain heteroatom. Naphthalene is the simplest example. PAHs occur in oil, coal, and tar deposits, are produced as byproducts of fuel burning. They are potent atmospheric pollutants. Some compounds have identified as carcinogenic and mutagenic. PAHs are also found in cooked foods for such as in barbecuing and in smoked fish. Our research interest is on Benzo[a]pyrenewhich is a PAH compound produced from incomplete combustion of coal tar, cigarette smoke, charbroiled food and automobile exhausts at 300 -600 degree Celsius. Its metabolite are highly mutagenic and carcinogenic and is listed in group 1(Carcinogens to human) by the IARC (International Agency for Research on Cancer). In early period the chimney sweepers suffered from scrotal and skin cancer in 18th -19th century.Benzo[*a*]pyrene found in tobacco smoke was shown to cause genetic damage in lung cells that was identical to the damage observed in the DNA of most malignant lung tumors. (Denissenko et al, 1996)A study proved that Benzo[a]pyrene is not carcinogenic instead benzo[a]pyrene is activated to a

DNA adduct substance called benzo[*a*]pyrene -7, 8-dihydrodiol-9, 10-epoxide which is activated by a group of metabolizing enzymes of our body known as cytochromeP450 enzyme system specially CYP450 1B1 group. **The cytochrome P450** super family of monooxygenases (**CYP**) is a large group of enzymes that metabolize the oxidation of organic substances. Substrates of CYP enzymes include metabolic intermediates such as lipids and steroidal hormones and xenobiotic compounds such as drugs and toxic chemicals. CYPs are major enzymes involved in drug metabolism and bioactivation, accounting for about 75% of the total number of metabolic reactions. (Guengerich et al, 2008)Benzo[*a*]pyrene is a procarcinogen that is converted to carcinogen. The mechanism is that benzo[*a*]pyrenediolepoxide, molecule intercalate in DNA, covalently bonding to the guanine nucleobases. It disrupts the process of replication and induces mutations, which lead to cancer after exposure. There are indications that benzo[*a*]pyrenediol epoxide specifically targets the protective p53 gene. (Pfeifer et al, 2002)This gene functions as a tumor suppressor. By inducing G (guanine) to T (thymidine) transversions, there is a probability that benzo[*a*]pyrenediol epoxide inactivates the tumor suppression ability in certain cells, leading to cancer and mainly skin cancer. The cytochromesP450 enzymes constitute a super family of heme-containing enzymes that catalyze the metabolism of a variety of xenobiotic compounds. This is a series of steps in which activation of molecular oxygen by the heme group and a process involving the delivery of two electrons to the P450 system followed by cleavage of the dioxygen bond, producing water and an activated iron-oxygen species which reacts with substrates through a various mechanisms. Therefore in order to suppress the activity of these enzymes, we have found drugs to inhibit the activity of enzymes from Tulsi (blackTulsi specifically). Tulsi scientifically named as *Ocimumtenuiflorum*.It is an aromatic plant of the family *Lamiaceae* and native plant of south Asia. The plant is erect, shrub, much branched with hairy stems

and green or purple leaves that are strongly scented. *We* have introspected the active compounds present in Tulsi e.g. eugenol, caryophyllene, carvacrol, elemene, linalool and others which help to reduce the benzo[a]pyrene cytotoxicity. The enzymes CYP1B1 specially, will get inhibited and it cannot further activate benzo[a]pyrene and it will be excreted as such without converting into harmful carcinogen. In our present studies we have generated results through*in-vitro* as well as computational studies which have concluded that Tulsi‘s active compounds help in reducing the cytotoxicity by Benzo[a]pyrene. The active compounds inhibit CYP450 enzymes from binding to Benzo[a]pyrene, instead the active compounds bind to CYP450 active site and inhibit the activation. We have proved our statement by both *in-vitro* and *in-silico*analysis.

In this report, we have studied *in-silico* studies first to figure out the binding sites of Tulsi’s compounds with CYP450 enzymes by docking studies. The binding sites were then confirmed using *in-vitro* studies like MTT assay, DAPI staining, Thin liquid layer chromatography, NMR and EROD Assay.

REVIEW OF LITERATURE

The human body is bombarded with diversity of xenobiotics, both from natural and anthropogenic sources. Xenobiotics are the foreign substances to the human body and only can enter from external sources like antibiotics. The vast majority of xenobiotics that find their way into the human body are naturally occurring phytochemicals, few areman-made chemicals have received most attention, since the focus of regulation is largely confined to these. The bodyrecognizes the xenobiotics as foreign, potentially harmfulto its survival, and response is to protect. Humans as well as animals have potential to remove these xenobiotics from the body by a complex set of enzymes. A number of enzyme systems, capable of metabolizing xenobiotics have been identified and extensively investigated, the most important enzyme system is thecytochrome P450-dependent mixed-function oxidases, aubiquitous system of haem-thiolate enzymes encountered inalmost every human organ, but with the highestconcentration in the liver, which consequently functions asthecentre of xenobiotic metabolism, being capable ofcatalyzing both oxidation and reduction pathways.

BENZO [A] PYRENE AFFECTS HUMAN HEALTH:

The main potent agent causing adverse effect in humans is benzo[a]pyrene-7, 8-diol-9, 10-epoxide which is formed from the activation of benzo[a]pyrene. The cytotoxicity depends upon the place and route of exposure of this hazardous metabolite. Its main exposure sites may be by inhalation, ingestion and surface exposures on skin. This results in formation of tumor at the site of exposure like skin tumor, rashes, skin irritation, lung tumor and liver tumor. Non-carcinogenic compounds like B[a]P which if absorbed for a long duration and multiple times results in enhancing its carcinogenic ability. Oral exposure to this compound causes sterility in progeny after mother is exposed to it and developmental deformality in reduced fetus weight and death at later stage. The

oral exposure to mice has confirmed the above mentioned cases. It causes adenomas and papillomas when exposed through intragastric way for duration of three months. The carcinogenic dose was 33.3mg/kg/day. Warts and skin eruptions were reported by the dermal exposure ofBaP for 1 to 3 months. (http://www.environment.alberta.ca/)

CYTOCHROMEP450

NAMING SYSTEM OF THE ENZYME:

Gene coding for cytochrome enzymes are denoted as CYP, and CYP1B1 means of family 1, and subfamily B and the polypeptide chain is 1.

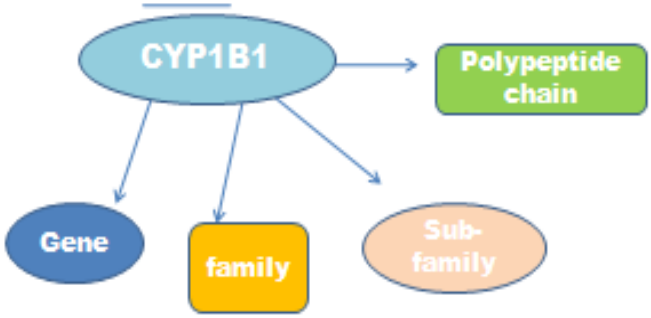


Fig1. Nomenclature of the enzyme

HISTORY OF CYTOCHROMES

Cytochromes P450 were found from the first experiment conducted by Axelrod and Brodie in the year 1955 .They identified the enzymes which were

responsible to oxidize xenobiotic compounds in the smooth ER of their liver. (Garfinkel et al, 1958) Another scientist Garfinkel and Klingenberg discovered that a CO binding pigment present in liver microsomes.(Omura and Sato et al,1964).These had an absorption spectra at 450nm which was named as Cytochrome P450.(Omura& Sato et al,1964) P450 is a low spin ferric heme containing proteins with a thiol residue as an heme ligand. (Bayer et al, 1969)

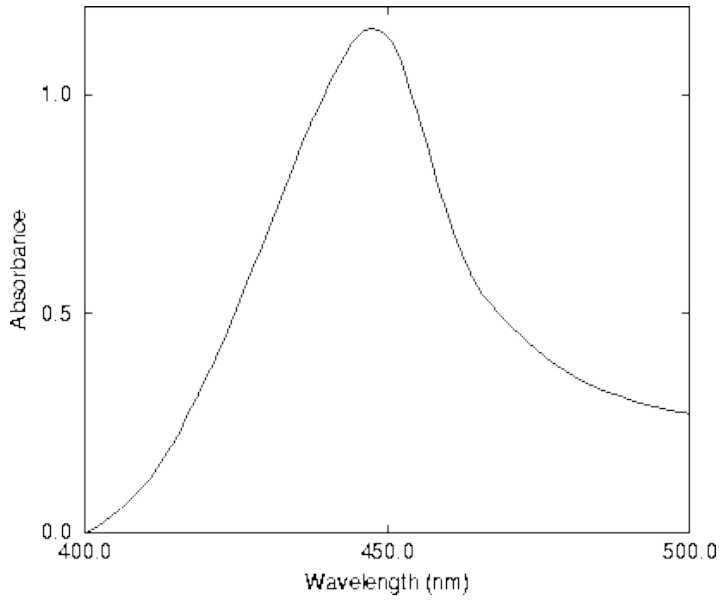


Fig.2. Absorption Spectroscopy of CYP enzymes

STRUCTURE OF CYTOCHROME P450

They contain an iron heme cofactor. The centre containing iron is bonded to porphyrin IX molecule and also two axial ligands(Shaik et al, 2004).The thiolate is one of the axial ligand derived from cysteine residues .The second ligand depends on the enzymatic reaction .But, originally water is bonded to the centre(Shaik et al, 2004). The valence shell of iron molecule contains i.e. two 4s electrons and in six electrons in 3d shell. The reaction of CYP occurs by converting the oxidation state of iron between Fe^{2+} and ferric Fe^{3+} (Lewis et al,

1996).The ferric state is more stable form. So, CYP450 enzymes get reduced with gain of electrons.

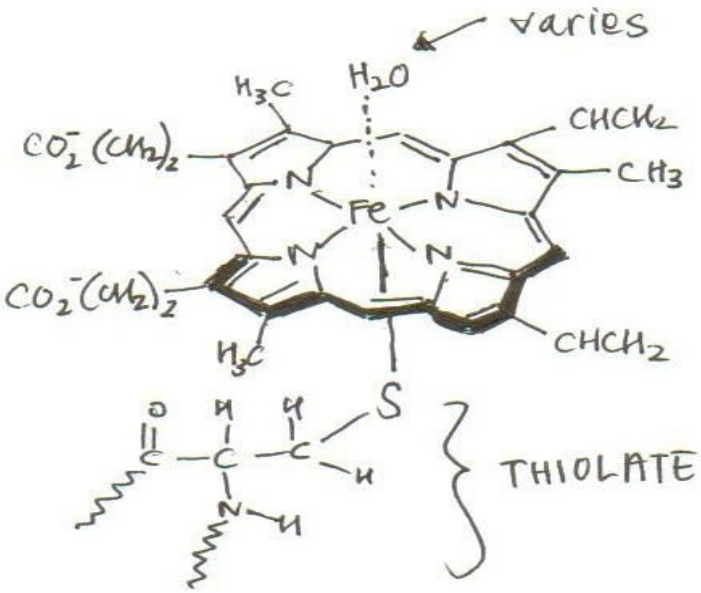


Fig.3. Structure of CYP450 enzymes

MECHANISM OF REACTION OF CYTOCHROMES:

The Cytochrome P450 catalysesmonooxgenase type of reaction by adding of one atom of oxygen to the aliphatic group of the substrate which results to water due to reduction of other oxygen atom.

$$\mathbf{RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+}$$

CYP1B1 is active in the metabolism of, benzo[a]pyrene and (7, 12-DMBA) (Hayes et al, 1996).

ROLE OF CYTOCHROME P450 ENZYMES:

CytochromeP450 enzyme system constitutes a super-family of heme-thiolate enzymes involving in the metabolism of exogenous molecules e.g. natural

products, drugs, and carcinogens and endogenous compounds e.g. hormones. The human cytochromeP450 enzymes are encoded by 57 genes and are divided into four classes. The Class I and Class II CYPs monooxygenases catalyzing a number of reactions such as conversion of alkenes to epoxides, alkanes to alcohols &arenes to phenols .(Sridhar et al, 2012) .The metabolism of carcinogens, procarcinogens, and chemotherapeutics by CYPs plays a important role in the cancer prevention and treatment strategies. Among all the different types of CYP 450 enzymes ,CYP1B1 was the mostly responsible in converting benzo[a]pyrene to 7,8-diol, benzo[c]phenanthrene further to 3,4-dihydrodiol, which is the precursors of the ultimate carcinogens.(Ioannides et al,2004).

STRUCTURE OF CYTOCHROME P450 ENZYME AND THEIR BINDING SITES:

The CYP1B1gene (Genbank accession no- U03688) is contained within 3 exons and 2 introns on chromosome 2p21 and spans 8.5 kb of genomic DNA.The catalytic domain consists of 543 amino acids which folds into a triangularprism shape (Fig.)(Sissung et al , 2006)additionally; there is a conserved sheet ofdomain near the N terminus of the protein. The number ofhelices is larger, but these helices are less conserved.Spatial conservation is largest for the structural core ofthe protein and diverges most for the substrate-binding site.Heme prosthetic group is the catalytic center of theenzyme.Most P450s are monooxygenases, and electrons for reductionof the heme and subsequently the oxygen substrate areprovided by protein partners that bind to the face of the proteinproximal to the heme. There are major six microsomal P450s (1A1, 1A2, 1B1,2E1, 3A4, and 2A6) which accountsfor most of the known carcinogenactivation pathways. Structures of 1B1 co-crystallized with theinhibitor alpha naphthoflavone (272 Da)(Wang et al, 2010).CYP1B1 are regulated by the Aryl hydrocarbon receptor. They are the Phase I reactions in drug metabolism.

FIG 4. CYP1B1 showing the inhibitor binding site i.e. alpha napthoflavone

TABLE 1.The table showed here reports all types of CYP450 enzymes responsible for bioactivation of PAH compounds

CYP 450 family	Human proteins	Role in carcinogenesis	Major characteristics of activated compounds	Principal characteristics
CYP1A	1A1,1A2	Very extensive	PAH, AA, HA, MC, NNK, AAB	Highly conserved; 1A1 extra hepatic, 1A2 hepatic

CYP1B	1B1	Very extensive	PAH, AA, HA, MC, NNK, AAB, Oestrogens	Over expressed in human tumors, skin cancer
CYP2A	2A6, 2A7, 2A13 2A1, 2A2, 2A3	Moderate	NA	Species differences in substrate specificity.
CYP2B	2B6 2B1, 2B2, 2B3, 2B12, 2B15, 2B16	Moderate	NA	bioactivation of carcinogenesis
CYP2C	2C8, 2C9, 2C18, 2C19	Moderate	Minor PAH	Important role in drug metabolism
CYP2D	2D6	Poor	NNK	Polymorphically expressed
CYP2E	2E1	Extensive	NA , HH	Propensity to generate reactive oxygen species
CYP3A	3A4, 3A5, 3A7	Moderate	PAH,MC,PA	Most important in drug metabolism

ACTIVATION OF BENZO [A] PYRENE BY CYP 1B1:

CYP1B1 is a unique enzyme that is expressed in many tumor types relative to normal tissue. It has the ability to activate a number of carcinogens in the chemical classes of PAHs, heterocyclic, aromatic amines, and nitro polycyclic hydrocarbons. Current studies has shown that CYP1B1 is involved in activation of environmental pro-carcinogen i.e. benzo [a]pyrene todiol epoxide which are carcinogen metabolites which forms DNA adducts. The first step in activation

of benzo [a]pyrene is formation of BaP- 7, 8- epoxide. Then followed by hydrolysis of BaP- 7, 8-epoxide to (–) – benzo[a]pyrene -7R-Trans-7, 8dihydrodiolmetabolite by microsomal epoxy hydrolase. This metabolite is further reacted by P450 to mutagenic benzo[a] pyrene-r-7, t-8-dihydrodiol-t-9, 10-epoxide species which is also known as diol epoxide 2 (DE 2). DE 2 is extremely reactive species which can bind to DNA, RNA and protein. It is reported that the DE 2 forms DNA adducts in the p53 tumor suppressor gene. (Sutter et al, 1998)

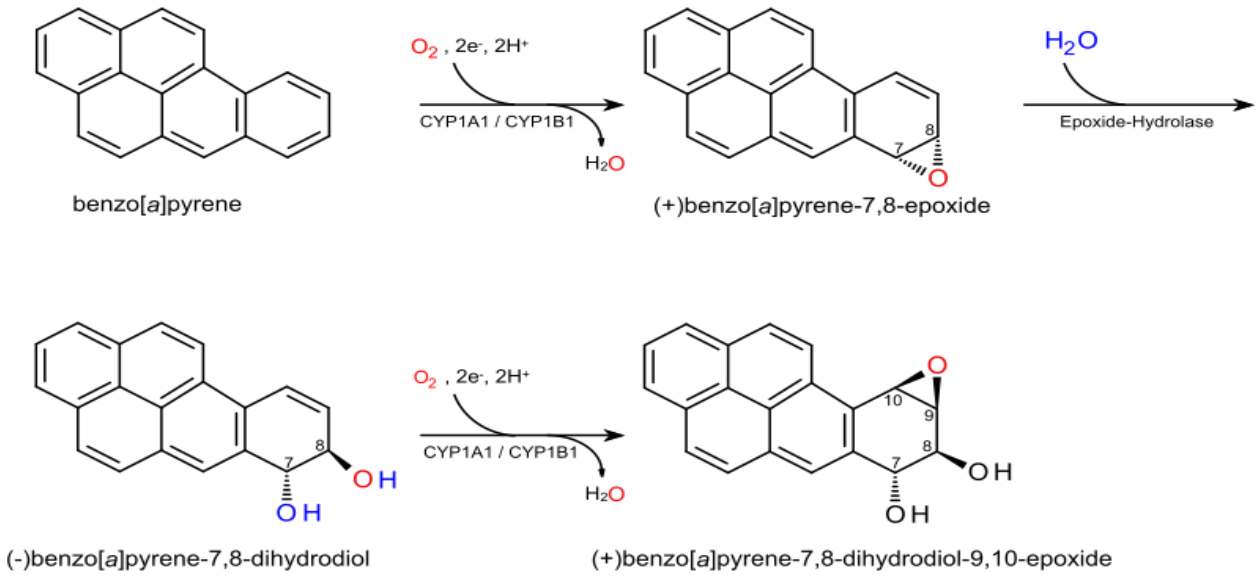


Fig .5 Bioactivation of benzo[a]pyrene by CYP1B1

INHIBITION OF CYP1B1 BY A NATURAL DRUG i.e. TULSI:

Commonly known as Tulsi or Holy Basil in English

Fig.6 BlackTulsi

Table 2. Classification of the plant

Kingdom	Plantae
Divison-	Magnoliophyta
Class-	Magnoliposida
Order-	Lamiales
Family-	Lamiaceae
Genus-	Ocimum
Species-	sanctum, tenuiflorum,gratissimum

Tulsisymbolizes piousness. It is considered as the holy plant in the Indian tradition rituals. It is found in almost all Indian homes for religious and medicinal purposes. They are mainly consists of two types of different morphology as seen in fig .7 one is with purple leaved or darker in color commonly known as Shyama or Krishna Tulsi.Another one is green or light colored leaves known as Rama tulsi .A third type of tulsi is also known to be growing as a forest tulsi known as Vanatulsi.

Fig 7.Two types of Tulsi: Krishna Tulsi& Rama Tulsi

TheTulsikavacham as described Tulsi as a protector of life of human-being till death (Dymock et al, 1993).So, the medicinal use of Tulsi is well documented to long back. All the parts of Tulsi are known to have used for therapeutic purposes such as the juice of the leaf is used eye drop preparation it also treats from chronic fever,dysentery,dyspepsia and hemorrhage (Uma Devi et al, 2001). The aqueous decoction of leaves of tulsi is given to patients suffering from gastric and hepatic disorders (Gupta et al, 2002).It is also used as a prophylactic against malaria with combination with black pepper. The seeds are reported to posses'anti-inflammatory, hypoglycemic, hypouricemic and uricosuricproperties. Experimentsof alcoholic and aqueous extract of tulsi 's leaves have the medicinal properties like antipyretic,analgesic, anti-asthmatic,diaphoretic, hypotensive, hypolipidemiaand anti-stress etc.(Sarkar and Pant,1989).The oil extracted from Tulsi has anti-bacterialand anti-oxidant properties which is used in industry of skin cream formation.(Singh et al, 2007).Tulsi due to its diversities of medicinal properties is known as Elixir of Life (Kumar et al ,2012).Cancer is the leading disease causing death world wide.Therefore all types of possible drugs from ethanobotany are experimented to show its anti-cancer properties. Tulsi was successfully documented as anti-

cancer agent at non-toxic concentration(Uma Devi et al,2001).Several constituents of Tulsi reported as active compounds which behave as anti-cancer agent were Eugenol, Caryophyllene,Flavanoids,Linalool, Carvacrol&Elemene.Eugenol is the major active constituent along with others. These components found effective against inhibiting Cytochrome P450enzymes from binding benzo[a]pyrene. These compounds bind to Cytochrome P450 inhibitory site.

OBJECTIVES

- **Objective 1**.

  Selection of inhibitor drugof cytochrome p450

- **Objective 2.**

  Preparation of both dry powder and liquid extract of tulsi and study the cytotoxicity mediated by benzo [a]pyrene.

- **Objective 3**.

  Studying the various in vitro studies of the inhibitor of cyp450 in HaCaT cells

- MTT assay
- DAPI staining
- EROD assay

- **Objective 4**.
  - Confirming the inhibitor of the cyp450 by in silico approach
  - Finding the ligands and receptor ‘s structure from Chem spider and protein data bank respectively
  - Studying the docking aspects of ligands with the receptor.

MATERIALS AND METHODS

In this report, my objective is to inhibit the activity of Cytochrome enzymes using a certain natural drug as inhibitor. For this purpose I have chosenTulsi as a natural drug playing the role of inhibitor.

SAMPLE COLLECTION:

The leaves of Tulsi were collected from the local areas which were known as black Tulsi .The leaves were washed throughrouly and kept for drying for 4-5 days at room temperature.

PREPARATION OF AQUEOUS EXTRACT AND DRY POWDERED FORM:

The sample was 14 gm in total. We distributed in two equal portions to prepare the extract and powder.

Aqueous Extract

- First dried leaves were weighed.
- Then to it 5 ml of distill water was added and grinded by mortar and pestle finally 30 ml more water was added to make the final extract.
- The paste was distributed equally two falcon tubes and centrifuged at 12000 rpmat 4 ° C for 20 mins.
- The supernatant was extracted and filtered by Whatman Paper and stored at -20 ° C

Dry Powdered

- Leaves were dried in vacuum evaporator.

- The dried leaves were made to powdered form by mortar and pestle.
- The powdered was grinded till it becomes finely grinded particles.
- Kept at 4 degrees.

In-vitro **analysis:**

CELL CULTURE:

In this experiment we are using HaCaT cell lines which are immortal human keratinocytes .All cells were grown as adherent monolayer in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10%v/v FBS (Fetal Bovine Serum) containing penicillin and streptomycin in a (5%) CO_2 incubator set at 37°C.

Procedure:

Once cells were confluent to 80-90% in healthy condition, they need to be passaged using trypsin for experiments. Before adding 0.5ml of 0.25% trypsin, cells need to be washed with 1x PBS-EDTA for reducing the serum effect, then the cells will easily detach by keeping them in 37°Cincubator for 3-5 min. After all the cells are detached the trypsin effects are neutralized by adding 1ml of serum containing media and repeated pipetting the cell suspension. Then aspirate the flask solution and collect in the micro centrifuge tubes to spin down at 1000 - 2500 rpm for 4-5 min. The pelleted cells were re-suspended in 1 ml of fresh medium and equally distributed in flasks containing complete medium. When seeding for experiments, live cells were stained by Trypan blue and counted using a haemocytometer.

MTT ASSAY:

It is a colorimetric assay for assessing viable cells. It measures the cell viability and proliferation in response to external factors

Principle:

MTT -3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide is a yellow color reduced to purple formazan in the mitochondria of living cells. The absorbanceof this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer.This reductiontakes place only when mitochondrial reductaseenzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. The formazan product of the MTT Tetrazolium accumulates as insoluble precipitate .This product must be solubilized prior to the absorbance readings. It is solubilized by DMSO.

Procedure:

- The MTT stock solution was made 5mg/ml MTT in PBS Solution .Then filtered by 0.22 mm filter.
- The HaCAT cells were seeded at the rate of 50000 cells/ml in 96 well plate for overnight incubation at 37°C and 5% CO2 in incubator.
- The cells were treated at different concentrations of benzo[a]pyrene i.e. at 0.5, 1, 2.5,5, 10 µM.
- On 72 hrs20µl of MTT was added to each well and incubated for 4 hrs.
- After 4hrs discard the media and 200 µl DMSO (Sigma) was added.
- The reading was taken by ELISA Plate reader at 595nm (Perkin Elmer).
- Similarly ,MTT with drug that is Tulsi + Benzo[a]pyrene was done at various concentration of Tulsi (10,20,50,100µg concentration)

DAPI NUCLEAR STAINING:

DAPI (4', 6-diamidino-2-phenylindole) is a nuclear stain that binds strongly to minor groove at A-T rich regions in DNA. It is used to see the nuclear damage in fluorescence microscopy. When bound to DNA, it has an absorption maximum at a wavelength of 358 nm and its emission maximum is at 461 nm which is blue in color. It also binds to RNA

- Drug treated preseededcells are treated with 4% paraformaldehyde
- After 8 hrs exposure then washed with PBS.
- Then DAPI stain was added to it
- Incubated for 5 mins. And observed under florescence microscope.

EROD ASSAY:

EROD (Ethoxyresorufin-0-deethylase) is a compound used to measure the activity of CYP450 enzyme (Klotz etal, 1984). This enzyme‘s activity converts ethoxyresorufin to resorufin and the assay is well known as EROD assay.

Steps:

- The cells were seeded at 50000 cells /ml
- Next day the cells reached the 70% of confluence
- Then drug i.e. tulsi was added in three different concentration 100 ,50 and 10µg in each well
- Add Benzo[a]Pyrene at concentrations 5,2.5 and 1µM
- Also add Tulsi and benzo[a]pyrene at concentration 100µg,5µM ,another 50µg,2.5µM and 10µg,1µM respectively indifferent wells
- Then the cells are washed with PBS and 100µL of EthoxyResorufurin was added.
- Reading was taken at 530 nm wavelength by Elisa plate reader.

The Extract of Tulsi is checked for the presence of active compounds by performing TLC (Thin Layer Chromatography)

Thin layer Chromatography (TLC)

It is a type of chromatographic process of separation of non-volatile mixtures of a sample (Lewis et al ,1989).It is done on glass, plasticor aluminum foil coated with silica gel ,cellulose and other material which act as adsorbent material.After putting the sample from 1.5 cm above from the edge of the bottom of the foil, the sample runs upwards because of capillary action due to different components present in t at different separation rate.

Steps:

- The dry powder of 1mg Tulsi was dissolved in 500µl methanol
- And dried in vacuum evaporated
- Place the silica gel foil in 3ml of n-Hexane and 7 ml of ethyl acetate.
- Then TLC was done
- Then it is seen under UV

Further to confirm presence of other components, NMR was performed.

***In-silico* Approach:**

For our in-silico approach we have use various bioinformatics tools like Chem Spider, PDB (Protein Data Bank and software's like Chimera, Open Babel, Hex6.3 and Ligplus.

STEP .1

In the first step structures of active compounds structure i.e. ligand structure files of eugenol, linalool, caryophyllene, carvacrol and elemene are retrieved from Chem spider.

CHEM SPIDER

Chem Spider is a chemistry database which is owned by the Royal Society of Chemistry and includes chemical information of more than 25 million compounds. It includes images of structures of 2D, 3D and articles (Pub med, Google books, scholar and journals).Every compound is denoted by individual identifiers (CAS Registry Number, InCHl.) The properties of those chemicals are also included. We have basically three types of searches- simple search, structure search and advanced search.

Steps:

- First entered the name of the ligands in the structure and simple search icons.
- Then the compound information page will appear which will consists of general name of the compound, Chem spider ID, molecular formula, monoisotopic mass, systematic chemical name and structure both in 2D & 3D.
- So, we can download the structures.

STEP.2

These structures which are retrieved are in molecular files (mol files). We have to convert those structures in PDB visualization. Software called Open Babel which helps in converting these mol files to pdb format

OPEN BABEL

It is a free and open source version of the Babel Chemistry File translation program. It is a project designed to interconvert between many file formats used in docking and molecular modeling.

Steps:

- We have selected the input file from the list after opening the software.
- Selected the file and the file format is displayed in the text box.
- We have selected the output format as .pdb
- Click on the Convert icon
- Save the file.

STEP.3

The receptor enzyme structure of cytochromeP450 is retrieved from PDB ID: 3PMO

PROTEIN DATA BANK (PDB)

The PDB (Protein Data Bank) is the worldwide archive of Structural data of Biological macromolecules, established in Brookhaven National Laboratories (BNL). It contains Structural information of the macromolecules determined by X-ray crystallographic, NMR methods etc. RASMOL [Raster Display of Molecules] is a molecular graphics program intended for the structural visualization of proteins, nucleic acids and small biomolecules. The program reads in molecular coordinate files and interactively displays the molecule on the screen in variety of representations and color schemes. All protein structure is retrieved from protein data bank and further applies for docking.

STEP .4

In this step the pdb file of CYP1B1 is obtained and it is bind to an inorganic inhibitor alpha –Napthoflavone. As we know alpha Napthoflavone is the inhibitor of CYP1B1(Wang et al , 2010) .For our studies we have to remove this inhibitor from the structure because we have to prove whether the active compounds of Tulsi act as inhibitor to CYP1B1.Therefore we used a software named Chimera for removing the inhibitor alpha - Napthoflavone .

UCSF Chimera 1.6.2-

UCSF chimera is highly extensible program for interactive visualisation and analysis of molecular structure and related data, including density map, supra molecular assembly, sequence alignment, docking results, trajectories and conformational ensembles. Chimera is highly extensible program for interactive visualization and analysis of molecular structures.

Steps:

- Inhibitor Ligand i.e.ANF is selected
- Ligand is removed

STEP.5

As we have removed the inhibitor ligand which was present in the active cavity of CYP1B1, the structure of CYP1B1 may be distorted and unstable. Hence the Software Swiss Pdb Viewer is used to repair the distorted geometries through energy minimization. In this implementation, all computations are done in vacuum, without reaction field

SWISS PDB VIEWER (SPDV)

Swiss pdb viewer is the software used to mutate the amino acid residue in the chain, showing the various bonds present in the structure, changing the orientations and energy minimizations etc.

STEP .6

Docking

Docking is done by Hex 6.2 software which is an interactive molecular graphics program for calculating and viewing feasible docking models with Evalue.

HEX 6.3

Hex is an Interactive Molecular Graphics Program for calculating and displaying feasible docking modes of pairs of protein and DNA molecules. Hex 6.3 can also calculate Protein-Ligand Docking, assuming the ligand is rigid, and it can superpose pairs of molecules using only knowledge of their 3D shapes .It uses Spherical Polar Fourier (SPF)correlations to accelerate the calculations and its one of the few docking programs which has built in graphics to view the result.

Steps:

- Go to file menu and Open the Receptor CYP1B1
- Again go to file menu and open ligand like eugenol.pdb, similarly other active compounds also in next docking process.
- We have to check the docking criteria of the both protein and the ligand .So, we have to fix various parameters.Then go to Control menu and adjust the SPF Transform, FFT steric scan, FFT final search ,MM refinement and finally FTR value
- Click for docking

STEP.7

After docking, the residual binding is analyzed by Ligplus software whether to check various interactions of the molecules. Interaction like hydrophobic, hydrogen bonding and vanderwaals forces are exerted on the residual binding.

LIGPLOT:

The active compounds bonded to the active sites or not were checked by this software. Then the active compounds were made to bind with benzo[a]pyrene.

RESULT AND DISCUSSION

1. *In- vitro studies*:

Since from *in-silico* studies it is confirmed that the compounds of Tulsi binds to CYP1B1.So by performing these in- vitro experiments MTT assay, DAPI and EROD assay we can confirm our objectives.

1.1 MTT ASSAY

a) Cells were treated with benzo[a]pyrene, and then cell viability was measured by MTT assay. B[a]P reduces the viability of HaCaT cells at indicated concentrations.Also in same plate the Tulsi were also added with B[a]P at indicated concentrations. The graph shows that Tulsi inhibits the cytotoxicity caused by Benzo[a]pyrene at dose dependent manner. From the figure below we concluded that lowest cell viability is seen when HaCaT Cells are treated with 5µM concentration of Benzo[a] Pyrene.

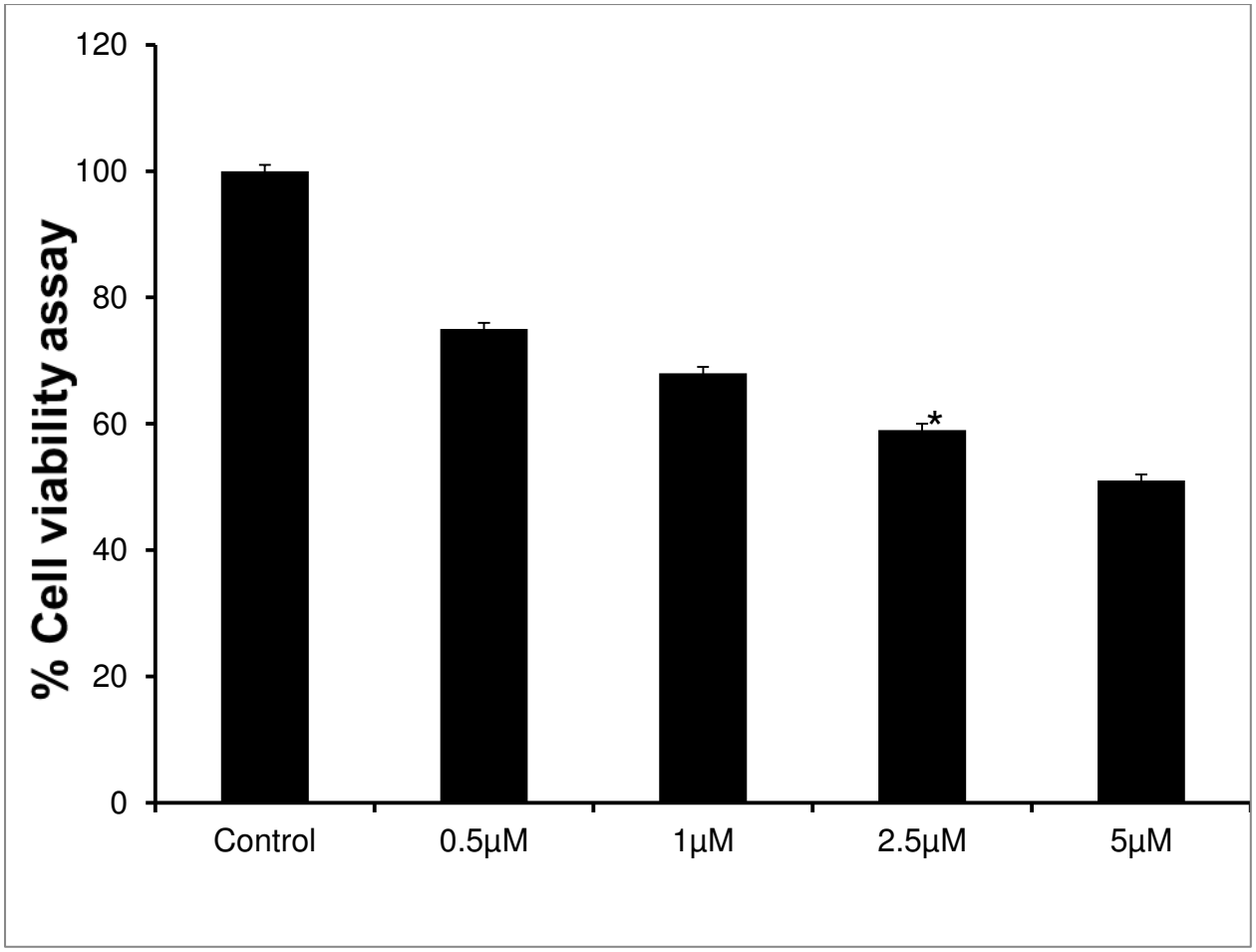


Fig no.8 The decrease in cell viability by increase in the concentration of B[a]P.

b)

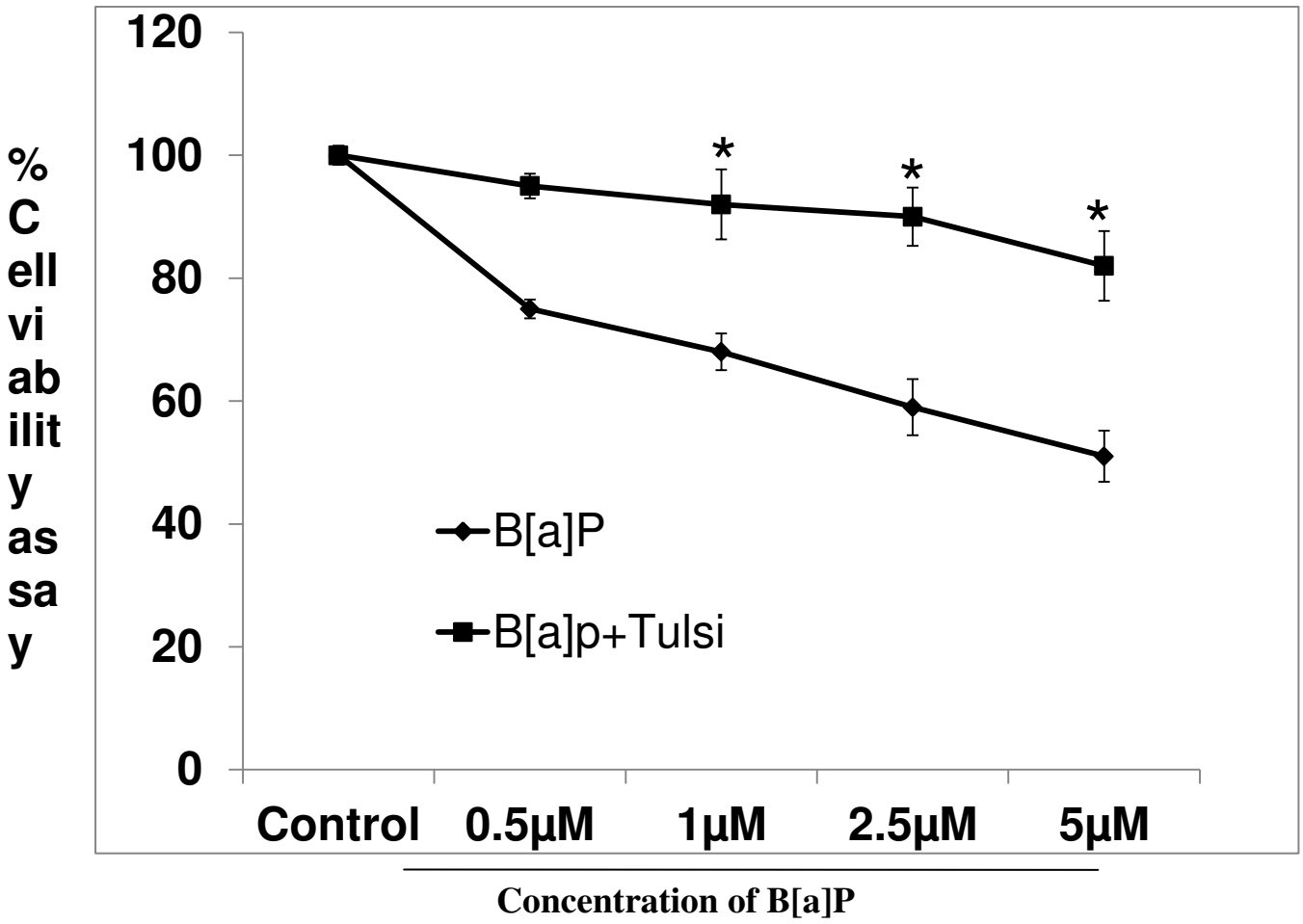


Fig no.9 It shows the comparison between cell viability when the cells are treated with B[a]P and B[a]P+Tulsi.

In the second MTT assay, it is performed on two aspects one is in the cells that are treated with B[a]Pand other aspect when cells are treated to B[a]P +Tulsi. It shows increase in cell viability upon treatment to Tulsi .Hence it confirms to inhibit cytotoxicity.

1.2 DAPI STAINING

In control we can see no DNA fragmentation was observed. As from the figure it is confirmed that at higher concentration of benzo[a]pyrene 5µM, DNA fragmentation and also membrane blebbing is observed. The dose dependent effect can be observed in the cells.

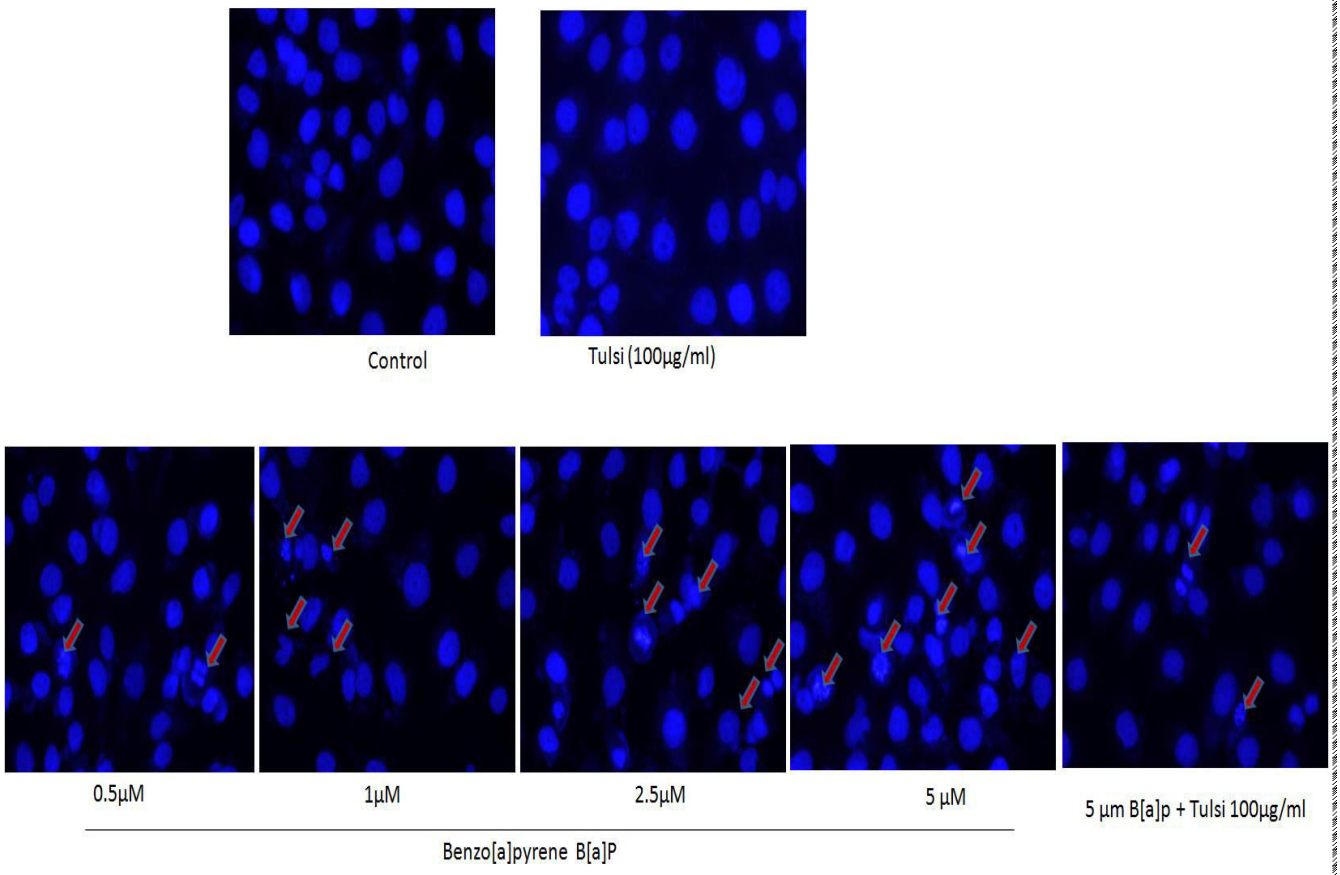


Fig. no.10 NoDNA damage is present on treatment with Tulsiand in B[a] P treated cells nuclear damage and membrane blebbing occurred

1.3 EROD ASSAY

The conversion of ethoxyresorufurin into resorufurin by liver enzymes confirms the activity of CYP450 enzymes. From this adding EROD to benzo[a]pyrene increases the activity of CYP450 enzymes. But, the EROD when added to Tulsi at different concentration normalize the activity of CYP450 enzymes .The Tulsi hence suppress the activity of CYP450 enzymes when added in combination of B[a] P at distinct differences shown from that of only B[a] P.

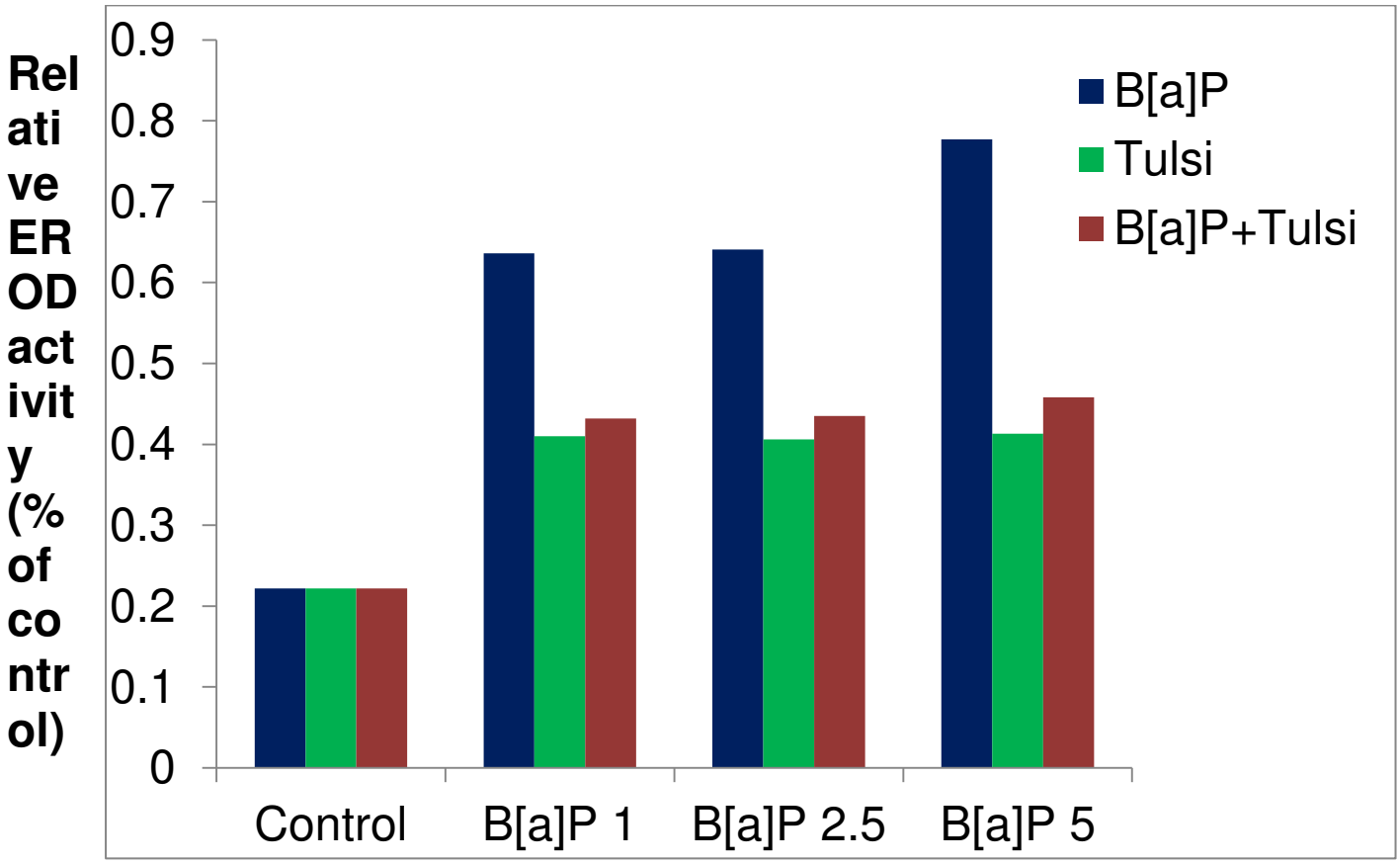


Fig no. 11 – The activity of cytochrome P450 enzymes on increasing Benzo[a]pyrene,Tulsi&Benzo[a]pyrene +Tulsi.

1.4. FROM TLC AND NMR:

The results of Thin Layer Chromatography was distinct four bands were seen clearly. From other sources it is confirmed that eugenol ,caryophyllene at R.F values of 0.7 giving orange brown and reddish violet color respectively.(

Referred From HCMS) Other bands were not identified .So , to further confirm the structures NMR Spectroscopy was done. The below mentioned figure is the NMR of Tulsi extract.

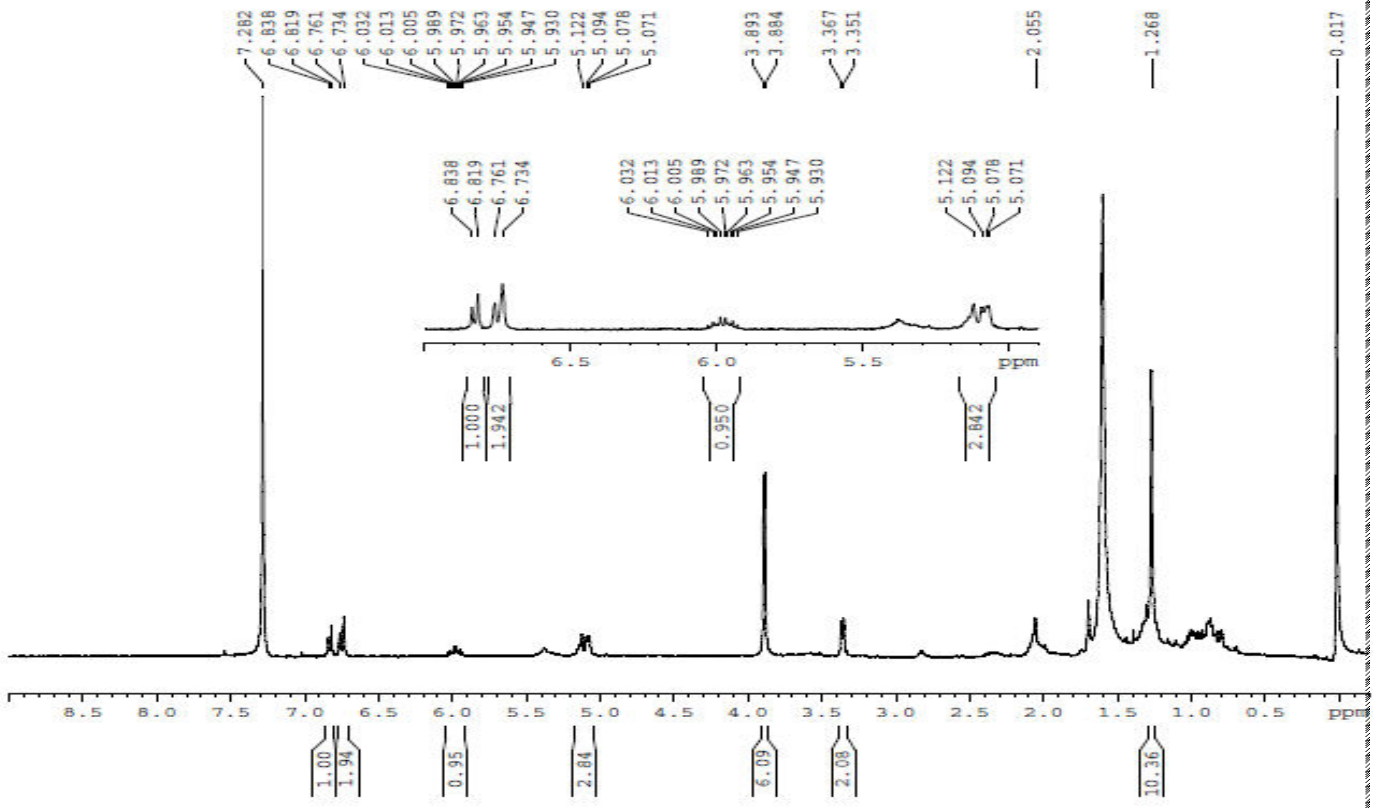


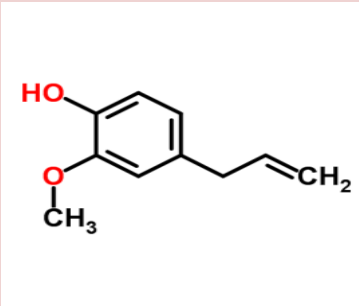
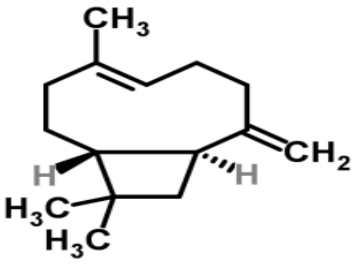
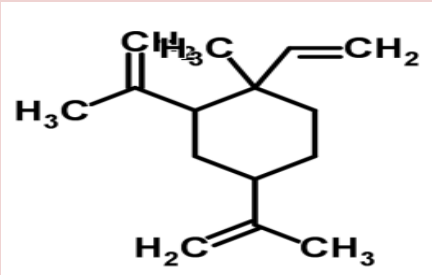
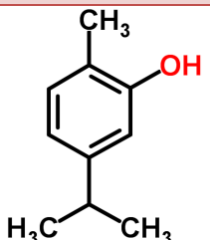
Fig no.12 The NMR Spectroscopy of Tulsi showing various constituents.

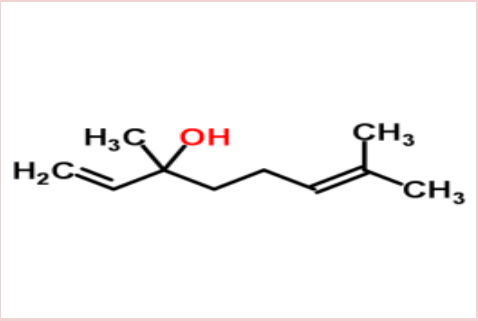
2. Insilco Analysis:

2.1 CHEM SPIDER:

The structures which were retrieved from Chem spider of the active compounds of Tulsi: It consists of the Chem spider ID, Systematic name, molecular formula and structure.

TABLE NO.3 - Structures of active compounds retrieved from Chem spider

Constituents of Tulsi (chem. spider ID) Systematic name	Molecular formula	Structure
Eugenol, 13876103 **[4-Allyl-2-methoxyphenol]**	$C_{10}H_{12}O_2$	
Caryophyllene,4444848 **[(1R,4E,9S)-4,11,11-Trimethyl-8methylenebicyclo[7.2.0]undec-4-ene]**	$C_{15}H_{24}$	
Elemene ,10138 **[2,4-Diisopropenyl-1-methyl-1-vinylcyclohexane]**	$C_{15}H_{24}$	
Carvacrol,21105867 **[5-Isopropyl-2-methylphenol]**	**C10H14O**	

Linalool ,13849981 **[3,7-Dimethyl-1,6-octadien-3-ol]**	**C10H18O**	

2.2 OPEN BABEL:

The above structures are converted to pdb format because the docking process would not understand the format of mol files.

2.3 PROTEIN DATA BANK:

The receptor Cytochrome 450 enzyme i.e. CYP1B1 structure was retrieved from PDB.

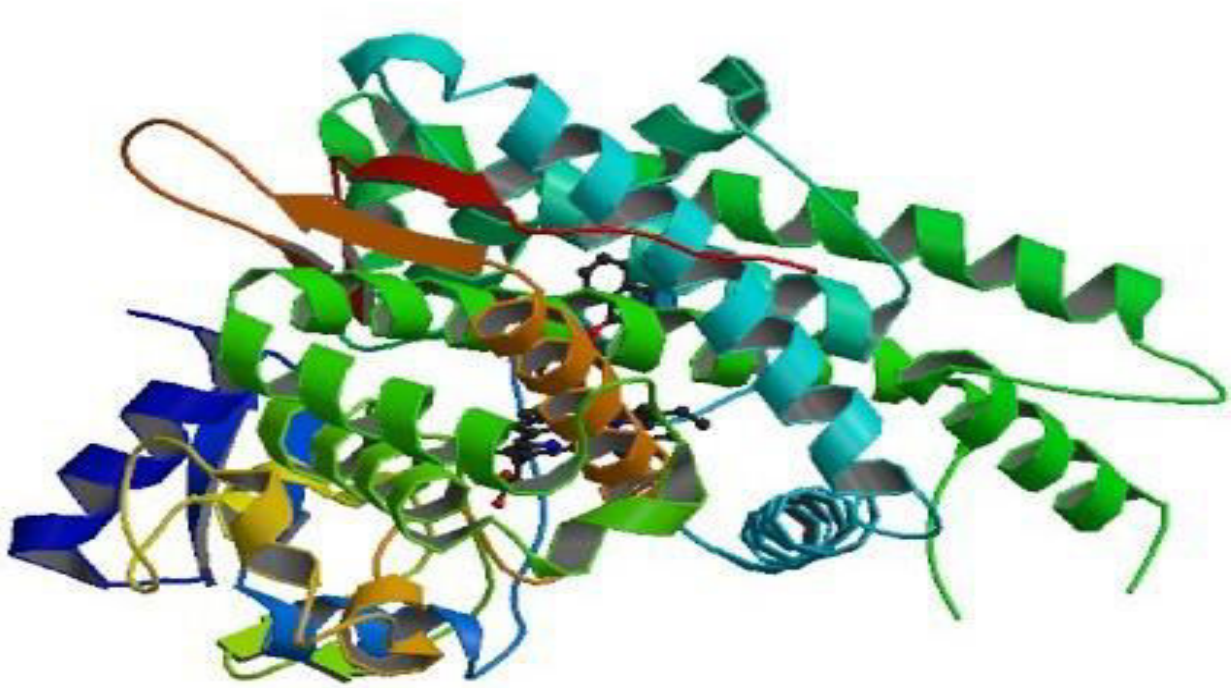


Fig no. 13 The structure of CYP1B1 with its inhibitor Alpha Napthoflavone from PDB.

2.4 UCSF CHIMERA:

The inhibitor alpha Napthoflavone was removed from the structure of CYP1B1.So that our active compounds can bind with CYP1B1.

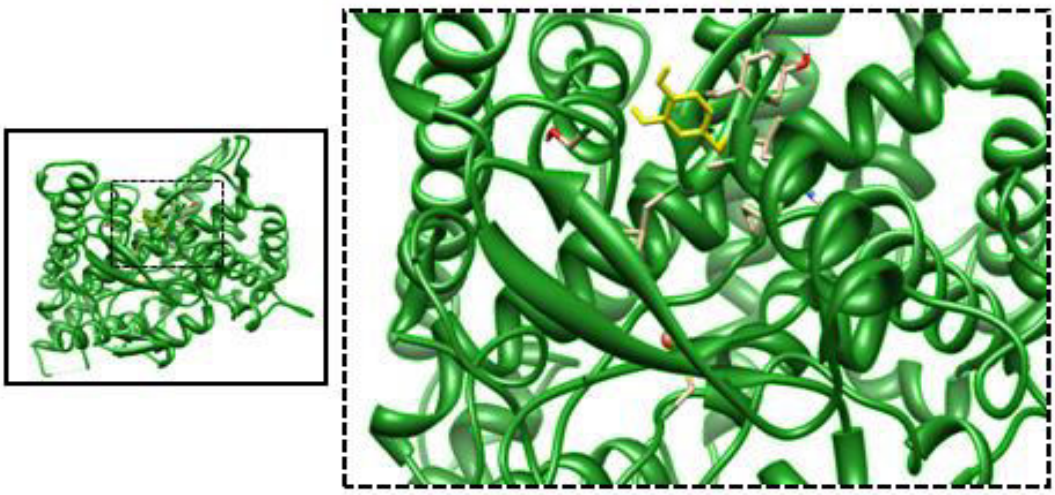
2.5 SWISS PDB VIEWER:

After removal of the structure of inhibitor present n CYP1B1, the structure was distorted. Hence the energy minimization was done by Swiss pdb viewer. The energy we get is -25794.668 KJ/MOL.

2.6 DOCKING STUDIES:

The activation of the CYP1B1 results in bonding to the active compounds of Tulsi. The high negative energies got through the docking with help of Hex 6.3 were confirmed that stable bonding at active sites. Further by software i.e. Lig plot, we confirmed that the active compounds of Tulsi do not bind to active sites of benzo[a]pyrene rather got bonded to non-specific binding. The table below shows the energies value of the binding with CYP1B1:

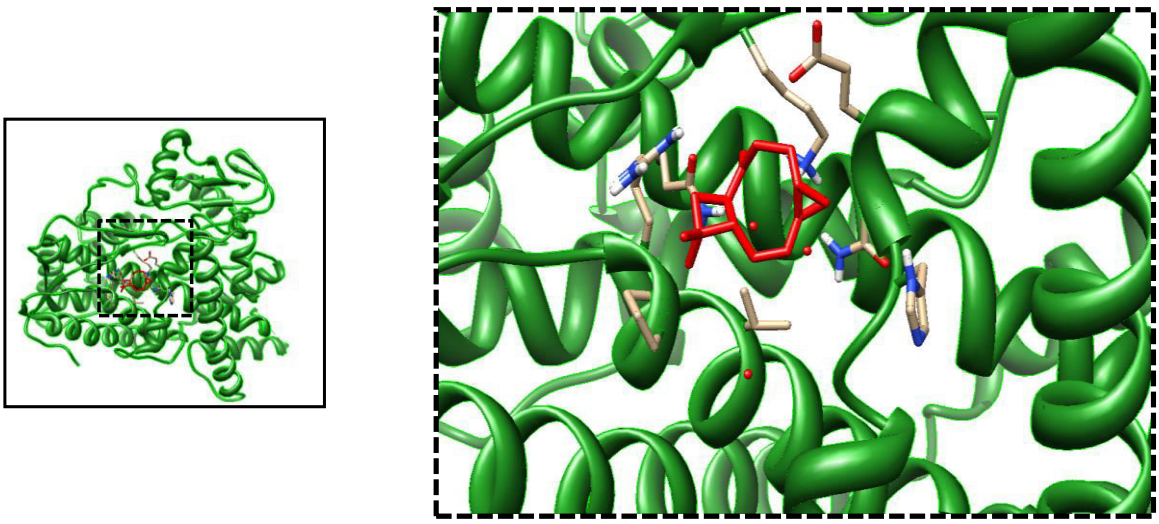
A)



CYP450-EUGENOL COMPLEX

Fig no.14 docking with eugenol and CYP1B1

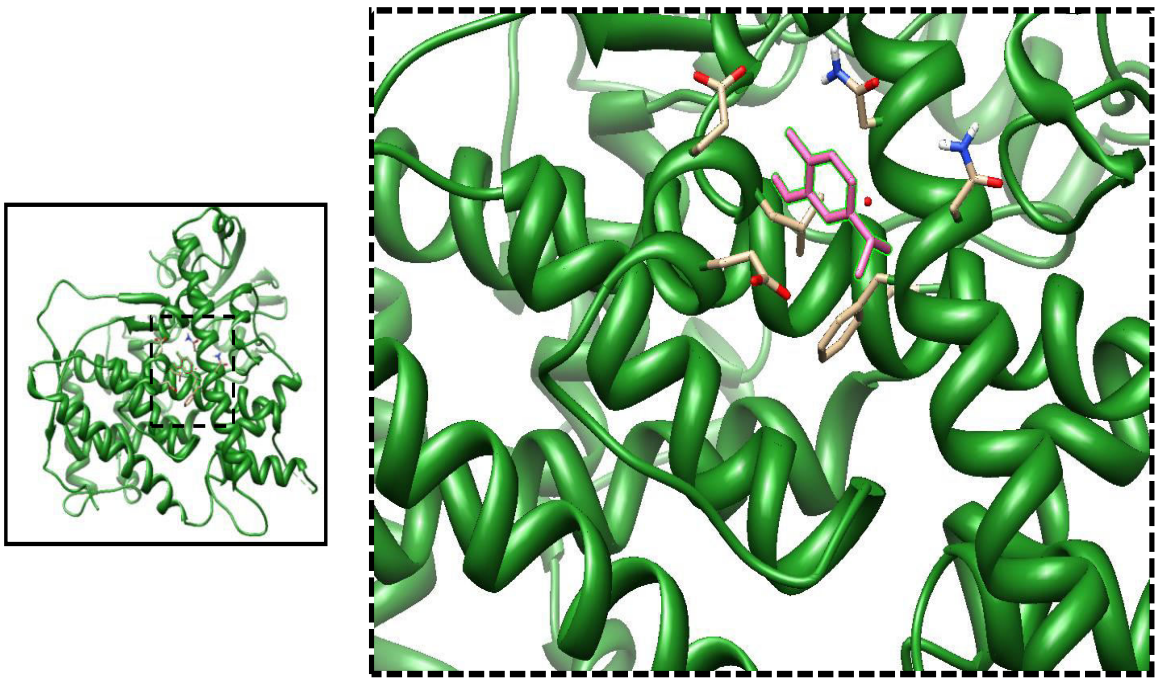
B)



CYP450-CARYOPHYLLENE COMPLEX

Fig no. 15 docking withcaryophylleneand CYP1B1

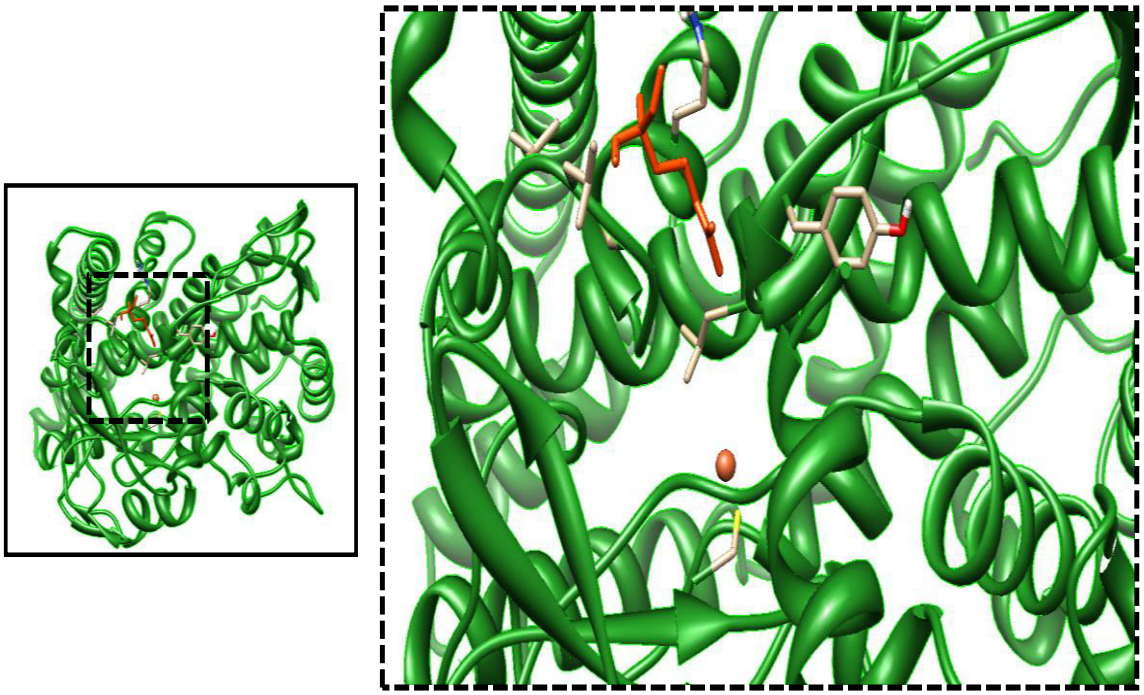
C)



CYP450-CARVACROL COMPLEX

Fig no.16 docking withcarvacrol and CYP1B1

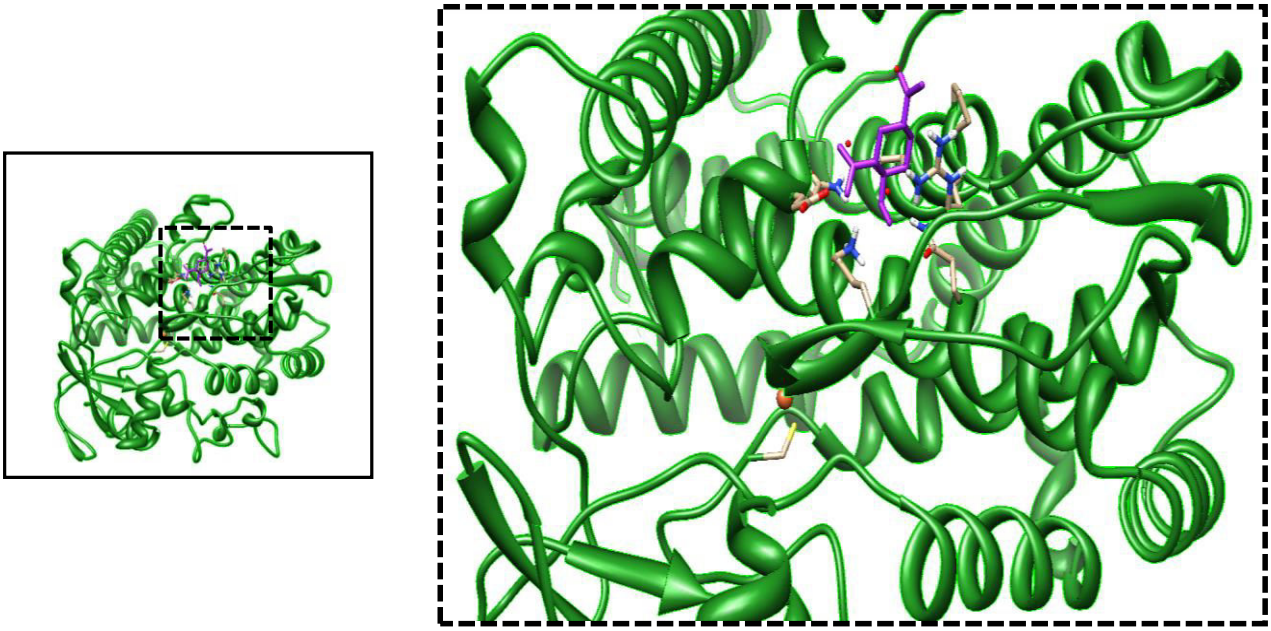
(D)



CYP450-LİNANOOL COMPLEX

Fig no 17 docking with linalool and CYP1B1

(E)



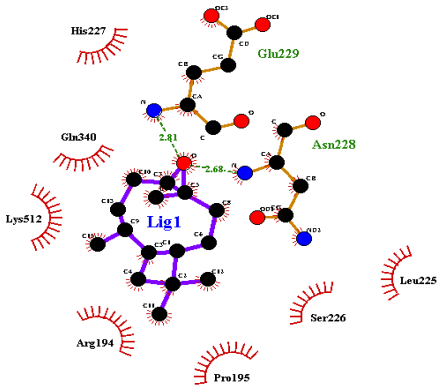
CYP450-ELEMENE COMPLEX

Fig no. 18 docking withelemene and CYP1B1

Table no. 3 the energies value of the binding with CYP1B1

CONSTITUENTS OF TULSI (CHEM. SPIDER ID)	BINDING ENERGIES WITH CYP (DOCKING E VALUE)KCAL/MOL
EUGENOL , 13876103	**-174.48**
CARYOPHYLLENE ,4444848	**-189.60**
ELEMENE ,10138	**-193.68**
CARVACROL ,21105867	**-165.5**

Ligand Protein Interactions by LIG PLUS:



cyp450caryophyllenecomplex

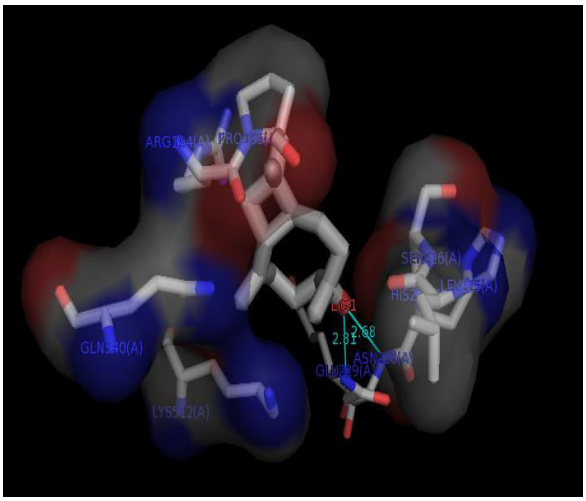
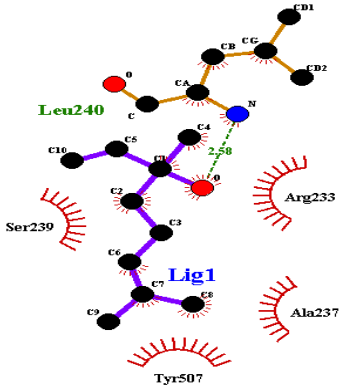


Fig. 19 Ligand protein interactions of caryophyllene



cyp450Linaloolcomplex

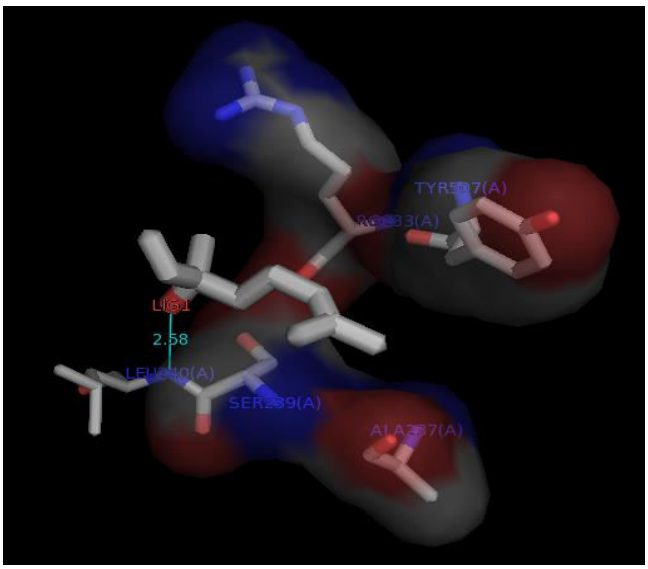
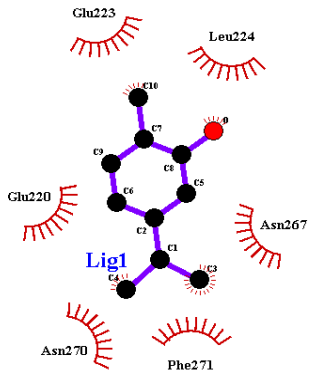


Fig.20 Ligand protein interaction of Linalool



cyp450carvacrolcomplex

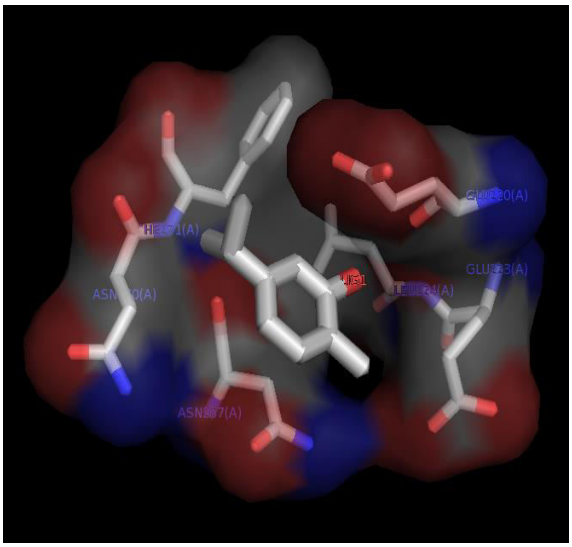
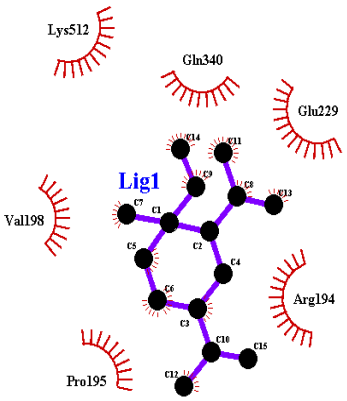


Fig. 21 Ligand protein interaction of carvacrol



cyp450Elemenecomplex

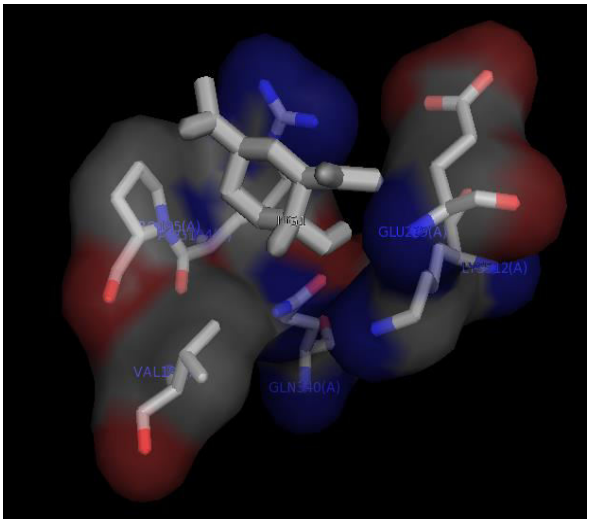
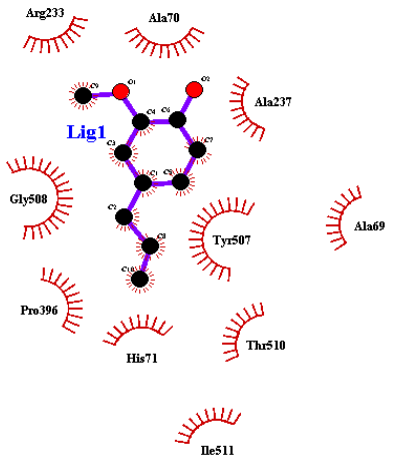


Fig. 22 Ligand protein interaction of Elemene



CYP450Eugenolcomplex

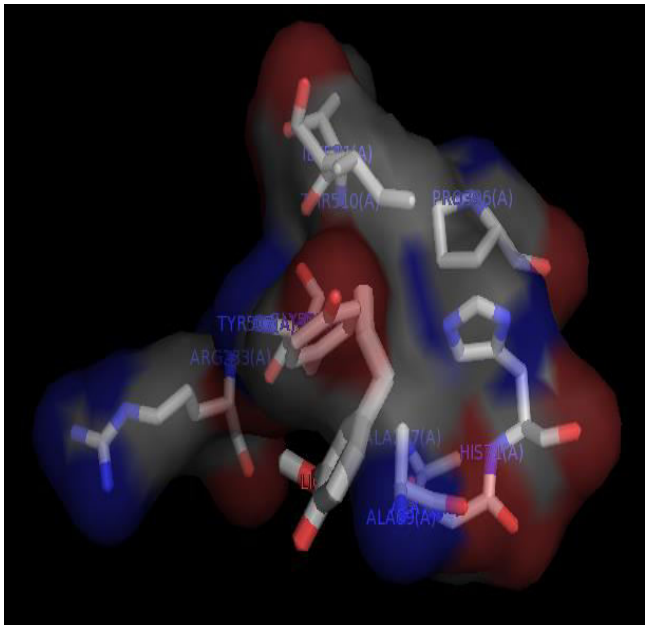


Fig. 23 Ligand protein interaction ofeugenol

CONCLUSION

From the above work, we have concluded that Benzo[a]pyrene causes cytotoxicity in HaCaT cells by DNA fragmentation and membrane blebbing through the DAPI experiments. Benzo[a] Pyrene causing cytotoxicity can be minimized by inhibiting CytochromeP450 enzymes. These enzymes are inhibited from binding to B[a]P by a natural drug Black Tulsi. It consists of many flavanoids, fatty acids, proteins and oils among all five major compounds confirmed from TLC and NMR that are present abundantly. They are eugenol, caryophyllene, carvacrol, elemene and Linalool .The active compounds present in Tulsi have shown high negative binding energies with CYP450 enzymes through docking studies. Tulsi unlike alpha naphthoflavone is more natural and free from side effects. Hence Tulsi can acts as powerful inhibitor towards CYP450 enzymes. From EROD assay it is confirmed that Tulsi inhibits CYP450 activity in general not specifically CYP1B1. MTT assay has also shown that the cells show more viability towards Tulsi's extract rather than benzo[a]pyrene. From our studies it shows all the active compounds like eugenol, caryophyllene, elemene, carvacrol and linalool have high reaction affinity to CYP450 enzymes.Interestingly, Tulsi has been evaluated inhibitor for Cytochrome P450 demonstrating blocking the activation of Benzo[a]pyrenewhich could have therapeutic benefits from several diseases including allergy and cancer.

REFERENCES

1. Kampa. Marilena, Castanas. Elias (2007). Human health effects of air pollution. Environmental Pollution 362-367
2. Schecter. A, Birnbaum. L, Ryan. J. J, Constable. J. D, (2006). Dioxins: an overview. Environmental Research 419-428
3. Ulrich. PoschlDr, (2005) Atmospheric Aerosols: Composition, Transformation, Climate and Health Effects. AngewandteChemie International Edition 7520-7540
4. Kampa. Marilena, Castanas .Elias (2007). Human health effects of air pollution. Environmental Pollution 362-367
5. Mastral. A .M, Callén. M .S, Murillo. R, (1996). Assessment of PAH emissions as a function of coal combustion variables. Fuel. Environmental Monitoring Assessment 1533–1536
6. Denissenko M .E, Pao.A, Tang.M-s. , PfeiferG.P. (1996) Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in P53. Science 430-432
7. Guengerich FP. (2008) Cytochrome p450 and chemical toxicology. Chemical Research in Toxicology 70–83
8. Gerd P Pfeifer, Mikhail F DenissenkoMagali Olivier , Natalia Tretyakova Stephen S Hecht and Pierre Hainaut (2002)Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. Oncogene 7435-7451
9. (http://www.environment.alberta.ca/)
10. GarfinkelD. (1958) Studies on pig liver microsomes. I. enzymic and pigment composition of different microsomal fractions. Archives of Biochemsitry and Biophysics 493-509

11. Omura .T and Sato .R.(1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. Journal of Biological Chemistry 0-8
12. Omura .T and Sato .R.(1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. Journal of Biological Chemistry 0-8
13. Bayer .E, Hill. H.A.O, Roder A. and Williams R. J. P. (1969.) The interaction between haem-iron and thiols. Chemical Communications 109
14. Shaik. S, S. P. de Visser, (2005). Cytochrome P450: Structure, Mechanism, and Biochemistry. Ortiz de Montellano, P. (ed.), New York: Kluwer Academic
15. Shaik. S, S. P. de Visser, (2005). Cytochrome P450: Structure, Mechanism, and Biochemistry. Ortiz de Montellano, P. (ed.), New York: Kluwer Academic
16. Lewis, D. F. V., (1996) Cytochromes P450: Structure, Function and Mechanism. Bristol: Taylor & Francis
17. Hayes CL, Spink DC, Spink BC, Cao JQ, Walker NJ, and Sutter TR (1996) 17 beta-estradiol hydroxylation catalyzed by human cytochrome P450 1B1. ProcNatlAcadSci USA 9776–9781
18. Sridhar Jayalakshmi, Liu Jiawang, Foroozesh Maryam and Stevens Cheryl L. Klein (2012) Insights on Cytochrome P450 Enzymes and Inhibitors Obtained
through QSAR Studies. Molecules 9283-9305
19. Ioannides Costas and Lewis David F.V.(2004) Cytochromes P450 in the Bioactivation of Chemicals. Current Topics in Medicinal Chemistry1767-1788
20. Sissung, T. M., Price, D. K., Sparreboom, A., and Figg, W. D. (2006). Pharmacogenetics and regulation of human cytochrome P450 1B1:

Implications in hormone-mediated tumor metabolism and a novel target for therapeutic intervention. Molecular Cancer Research 135–150

21. Wang An, SavasUzen , Stout C. David and Johnson Eric F.(*2010)*Structural Characterization of the Complex between -Naphthoflavone and Human Cytochrome P450 1B1.Journal of Biological Chemistry 5736-5743
22. Kim H. James, Stansbury H. Kelvin , Walker J. Nigel, Trush A. Michael , Strickland T.Paul ,Sutter R.Thomas .(1998) Metabolism of benzo[a]pyrene and benzo[a]pyrene-7,8-diol by human cytochrome P4501B1. Carcinogenesis 1847-1853
23. Dymock W, Warden, CHJ , Hooper D.(1893) Ocimumsantum , In PharmacographiaIndica – A History of principal drugs of vegetable origin .86
24. Uma Devi P. (2001) Radioprotective ,anti –carcinogenic and anti-oxidant properties of Indian holy basil ,*Ocimum sanctum* .Indian J. Exp. Biology 185
25. Gupta SK, Prakash J, Srivastva S. (2002) Validation of tradition claim of Tulsi, Ocimum Sanctum as a medicinal plant. Indian J Exp Biology 765
26. Sarkar.A and Pant M.C.(1989) A Comparative study of the Hypoglycemia action of the seeds and fresh leaves of Ocimum sanctum (Tulsi). Indian Journal of Physiological Pharmacology 197
27. Singh .S, Taneja .M, Majumdar DK (2007) Biological activites of ocimum sanctum fixed oil – an overview .Indian J. Exp. Bio. 1212
28. Kumar. P, Kumar. M. Kavitha .K, Singh Jagadeesh and Khan Rawoof (2012) Pharmacological Actions of Ocimum sanctum. International journal of advances in pharmacy, Biology and chemistry
29. Wang An, SavasUzen , Stout C. David and Johnson Eric F.(*2010)*Structural Characterization of the Complex between -Naphthoflavone and Human Cytochrome P450 1B1Journal of Biological Chemistry 5736-5743

30. Lewis W. Harry and Moody J. Christopher.(1989) Experimental Organic Chemistry: Principles and Practice 159-173
31. Klotz A.V., Stegeman J.J. and Walsh C. (1984) An alternative 7-ethoxyresorufin O-deethylase activity assay: a continuous visible spectrophotometric method for measurement of cytochrome P450 monooxygenase activity. Analytical Biochemistry 138-145.
32. http://www.hcms.org.in/product_detail.php?product=11